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(54) Title: MODULATION OF MAP KINASE PHOSPHATASE 1 (MKP-1)

(57) Abstract

A method of modulating the migration of vascular smooth muscle cells in a vertebrate is disclosed. The method includes the steps of identifying a vertebrate suspected of having a condition characterized by the migration of vascular smooth muscle cells, providing an agent that increases expression of MKP-1 in vascular smooth muscle cells, and administering to the vertebrate an amount of the agent sufficient to inhibit migration.

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MODULATION OF MAP KINASE PHOSPHATASE 1 (MKP-1)

Reference to Related Applications

This application claims priority from provisional application 60/030,927, filed November 15, 1996.

Background of the Invention

The invention relates to the treatment of vascular conditions.

Cellular adhesion and migration characterize many physiological and pathological processes, including embryogenesis, tissue morphogenesis, metastasis and vascular occlusion. Huttenlocher et al., *Curr. Opin. Cell. Biol.* 7:697-706 (1995); Lauffenburger et al., *Cell* 84:359-369 (1996). Vascular conditions such as post-angioplasty restenosis, transplant arteriopathy, graft stenosis following coronary bypass surgery and chronic atherosclerosis are all characterized by the adhesion and migration of smooth muscle cells (SMC).

Overexpression of Mitogen Activated Protein Kinase Phosphatase 1 (MAP kinase phosphatase 1, MKP-1) has been shown to inhibit the proliferation of SMC *in vitro*. Lai et al., *J. Clin. Invest.* 98:1560-1567 (1996). This effect may be due to the ability of MKP-1 to dephosphorylate MAP kinase, which becomes activated during SMC migration and proliferation. MKP-1 was originally identified as an immediate early gene in rat fibroblasts subjected to oxidative stress. Overexpression of MKP-1 has also been shown to inhibit the induction of the mesodermal bud, a process characterized by extensive cellular migration, in the Xenopus embryo. Gotoh et al., *EMBO J.* 14:2491-2498 (1995); LaBonne et al., *Development* 121:1475-1486 (1995).

MKP-1 is a member of the family of dual specificity tyrosine phosphatases, nuclear proteins which dephosphorylate both phosphotyrosine and phosphothreonine residues on target proteins. Substrates of MKP-1 and other dual specificity tyrosine phosphatases are limited to proteins with adjacent phosphorylated tyrosine and threonine residues. Such proteins include CDC2, CDK4 and the MAP kinases, all of which are involved in regulating expression of genes involved in cell growth. MKP-1 expression can be influenced by Ca^{2+}, angiotensin II, epidermal growth factor (EGF), platelet derived growth factor (PDGF) and H_2O_2. Duff et al., *J. Biol. Chem.* 268:26037-26040 (1995).

MKP-1 homologues have been identified in mouse (3CH134) and human (CL100). CL100 and 3CH134 are nearly identical (Ishibashi et al., *J. Biol. Chem.* 269:29897-29902 (1994)), and both dephosphorylate phosphotyrosine, phosphothreonine and phosphoserine on artificial substrates. Alessi et al., *Oncogene* 8:2015-2020 (1993); Charles et al., *Proc. Natl. Acad. Sci. (USA)* 90:5292-5296 (1993). The rat, mouse and human gene products are collectively referred to as MKP-1, because of their specificity for MAP kinases.

Summary of the Invention

The invention is based on the discovery that SMC, e.g., vascular smooth muscle cells, that differ in the level of MKP-1 expression exhibit altered cellular adhesion and migration. Administration of agents which alter MKP-1 expression or activity can therefore be used to treat pathological conditions characterized by SMC adhesion and migration.

The invention features a method of inhibiting the migration of vascular smooth muscle cells. The method includes the steps of:

identifying a vertebrate suspected of having a condition characterized by the migration of vascular smooth muscle cells;

providing an agent that increases expression or activity of MKP-1 in vascular smooth muscle cells; and

administering to the vertebrate an amount of the agent sufficient to inhibit migration of the cells.

The agent preferably inhibits migration by at least 20%, preferably 50%, more preferably 75% or 85%, and most preferably 95%.

In one embodiment, the agent is a nucleic acid sequence encoding MKP-1. A naturally occurring nucleic acid sequence may be either genomic DNA or a RNA transcript, such as mRNA.

The vertebrate is preferably a mammal, such as a human, a non-human primate, or a dog, cat, cow, pig, horse, sheep, goat, rat, mouse, guinea pig, hamster or rabbit. The condition being treated can be, for example, post-angioplasty restenosis, transplant arteriopathy, graft stenosis, or chronic atherosclerosis.

The invention also includes a screening assay for the detection of agents which increase MKP-1 expression or activity. The assay includes the steps of:

providing a cell-free preparation of MKP-1,

contacting the MKP-1 with a test compound;

detecting MKP-1 activity;

determining whether the test compound increases MKP-1 activity, wherein an increase in MKP-1 activity in the presence of the test compound indicates that the test compound is potentially useful for inhibiting migration of vascular smooth muscle cells. The test compound inhibits MKP-1 activity by at least 20%, preferably 50%, more preferably 75% or 85%, and most preferably 95%. MKP-1 activity can be detected using a substrate of MKP-1's phosphatase activity.

In addition, the invention includes a method for identifying a compound which inhibits migration of vascular smooth muscle cells. The method comprises the steps of:

providing a cell which expresses MKP-1;

contacting the cell with a test compound;

detecting MKP-1 expression in the cell; and

determining whether the level of MKP-1 expression is increased in the cell in the presence of the test compound, wherein an increase in MKP-1 activity in the presence of the test compound indicates that the test compound is potentially useful for inhibiting migration of vascular smooth muscle cells. The compound inhibits expression by at least 20%, preferably 50%, more preferably 75% or 85%, and most preferably 95%. MKP-1 expression can be detected using an antibody-based assay or a nucleic acid hybridization assay.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Brief Description of the Drawings

Fig. 1 is a bar graph showing the number of MKP-1 cells and control smooth muscle cells (SMC) adhering to collagen in an adhesion assay.

Fig. 2 is a series of sequential photographs showing cell spreading in individual MKP-1 cells and control SMC in response to collagen.

Fig. 3 is a bar graph showing the percentage of MKP-1 cells and control SMC spreading in response to collagen.

Fig. 4 is a bar graph showing the number of MKP-1 cells and control SMC migrating in response to collagen, in the presence and absence of platelet derived growth factor (PDGF).

Fig. 5 is a bar graph showing the number of 3T3/MKP-1 cells and control 3T3 cells migrating in response to collagen, in the presence and absence of PDGF.

Fig. 6 is a bar graph showing the effect of antibodies specific for $\alpha 2\beta 1$ or $\alpha 3\beta 1$ integrins on the migration of MKP-1 cells and control SMC in response to collagen.

Fig. 7 is a bar graph showing the number of MKP-1 cells, C258S/MKP-1 cells, and control SMC migrating in response to PDGF.

Fig. 8A-8E are Western blots probed with antibodies specific for tyrosine phosphorylated focal adhesion kinase (FAK) (A); FAK (B); paxillin (C); vinculin (D); and α-actin (E).

Fig. 9 is a series of photographs showing focal adhesion complexes in individual MKP-1 cells, C258S/MKP-1 cells and control SMC.

Fig. 10 is a series of photographs showing actin structure in individual MKP-1 cells, C258S/MKP-1 cells and control SMC.

Detailed Description

The invention is based on the finding that MKP-1 regulates cellular adhesion and migration. Overexpression of MKP-1 in smooth muscle cells (SMC) or NIH 3T3 cells results in increased cellular adhesion and migration in the presence of type I collagen. In contrast, overexpression of MKP-1 in SMC in the absence of type I collagen results in decreased migration of SMC towards platelet derived growth factor (PDGF). Administration of agents which alter MKP-1 expression or function can therefore be used to treat pathological conditions characterized by unwanted or aberrant SMC adhesion and migration. Conditions associated with unwanted SMC adhesion and migration for which increased levels of MKP-1 expression or function is desired include post-angioplasty restenosis, transplant arteriopathy, graft stenosis following coronary artery bypass surgery, and chronic atherosclerosis.

Materials and Methods

Transfection

SMC were transfected with vector pcDNA3 (Invitrogen) (control SMCs), or with pcDNA3/MKP-1 (MKP-1 cells) as described. Lai et al., *J. Clin. Invest. 98:1560* (1996) . C258S/MKP-1 cells have been transfected with a dominant negative mutant MKP-1 gene C258S, in which the catalytic residue cysteine 258 has been replaced with a serine in the plasmid pcDNA3/MKP-1 (Lai et al., *J. Clin. Invest. 98:1560-1567* (1996)). Transfections were performed by the Lipofectin (Life Technologies) method according to the manufacturer's instructions.

Adhesion Assay

Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 0.4% fetal calf serum and 300 μg/ml G418 for 24 hours. Trypsin-EDTA was added to the cultures, which were then incubated at 37°C for three minutes. The detached cells were washed once in DMEM containing 10% fetal calf serum and three times in phosphate buffered saline (PBS). After the final wash, the cells were resuspended in DMEM supplemented with 10% fetal calf serum and 300 μg/ml G418. The cells were then plated in 6-well tissue culture plates coated with 10 $\mu g/cm^2$ type I collagen for 24 hours. The number of adherent cells was calculated as the initial total plating number (100,000 cells) minus the number of nonadherent cells. Nonadherent cells in the culture medium were removed and combined with nonadherent cells which came off in three PBS washes of the adherent cells, and the number of nonadherent cells was counted on a Coulter counter. Similar results were obtained in two experiments.

Cell Spreading Assay

Cells were trypsinized, washed and plated as described above. After thirty minutes, the cells were photographed in 5-6 random fields at a magnification of 200x, and spreading cells were counted as described previously. Richardson et al., *Nature* 380:538-540 (1996). Briefly, spreading cells were defined as those cells that were not light reflective, while non-spreading cells were defined as those cells that were light reflective. Each cell in the field was counted, and the percentages of spreading and non-spreading cells were calculated by counting 150-200 cells. The standard deviations were derived from three separate experiments.

Migration Assay

A migration assay using a modified Boyden chamber was performed as described previously. Bornfeldt et al., *J. Clin. Invest.* 93:1266-1274 (1994). In order to generate a population of quiescent cells, SMC were incubated in DMEM supplemented with 0.4% calf serum and 300 μg/ml G418 for 48 hours. The bottom side of a polycarbonate filter (pore size 8 μm) was coated with 100 μg/ml type I collagen (Vitrogen 100) 24 hours before use. The bottom of the Boyden Chamber contained DMEM with various concentrations of PDGF, and the top contained 50,000 cells/well. The assay was carried out at 37°C in a tissue culture incubator. After four hours, cells adhering to the lower surface of the membrane filter of the Boyden chamber were fixed and stained. Cells which had not migrated were removed by mechanical scraping of the upper chamber. The filter containing the fixed and stained cells (migrated cells) were mounted on glass slides. Cell migration through the polycarbonate filter was assessed by counting the cells at a magnification of 200x.

In some PDGF-mediated migration assays, the experiments were performed as above, except that the polycarbonate filter was not coated with collagen.

In some experiments, the cells were treated with antibodies specific for either $\alpha 2\beta 1$ or $\alpha 3\beta 1$ integrin (Dako Corp.) prior to being loaded into the Boyden chamber. In these experiments, the cells were incubated for twenty minutes at room temperature with the antibodies, which had been diluted 1:50.

Northern Analysis

NIH 3T3 cells were stably transfected with pcDNA vector alone (3T3 control cells) or pcDNA3 with full length human MKP-1 (3T3/MKP-1 cells). These vectors

carry the gene for neomycin resistance. Cells which had taken up the vectors were selected in medium containing G418 (500 μg/ml) and single cells were cloned. Total RNA was extracted from quiescent cells grown from specific clones, and subjected to electrophoresis and Northern blotting. Quiescent cells were obtained by culturing the cells for 48 hours in quiescent medium (DMEM, 0.4% calf serum, 100 units/ml of penicillin, 100 μg/ml of streptomycin, 1% L-glutamine) using RNZol B Plus (Tel-Test Inc., TX). Blots were probed with ^{32}P-labeled random-primed human MKP-1 probe.

Immunoprecipitation and Western Blotting

Confluent cells (control SMCs and MKP-1 cells) were cultured in DMEM supplemented with 0.4% fetal calf serum and 300 μg/ml G418 for 24 hours. Cells were washed with PBS containing 1 mM sodium vanadate three times, lysed with RIPA buffer at 4°C for 10 min, and the cell lysate was passed through a 22 gauge needle five times. Debris was removed by centrifugation at 12,000 x g for 12 minutes. Supernatants were used for immunoprecipitation and Western blotting. Tyrosine phosphorylated FAK was immunoprecipitated from supernatants (500 μg protein) using agarose beads conjugated with anti-phosphotyrosine antibody 4G10 (UBI), and detected using anti-FAK antibodies (Santa Cruz) on ECL (Enhanced Chemiluminescence; Amersham) Western blots. FAK was immunoprecipitated from cell lysates (500 μg protein) by anti-FAK antibodies and protein G beads, and detected with anti-FAK antibodies on Western blots. Paxillin, vinculin and α-actin in cell lysate (20 μg protein/lane) were detected on ECL Western blots with anti-paxillin, anti-vinculin and anti-α-actin antibodies (Dako), respectively.

Focal Adhesion Complex Assay

In order to examine focal adhesion complexes, cells were plated on glass cover slips and cultured in DMEM supplemented with 2% fetal calf serum and 300 μg/ml G418. After a two day incubation, the cells were permeabilized in 0.2% Triton-X 100 in PBS, and fixed in 4% paraformaldehyde in PBS. Focal adhesion complexes were detected by immunostaining with rabbit anti-vinculin antibody (Sigma), diluted 1:100, followed by anti-rabbit Ig-rhodamine. Cells were examined and photographed under a fluorescence microscope at a magnification of 400x. Cells with polar focal adhesion complexes were defined as cells having more than 25% of their cell surface edges without focal adhesion complexes, i.e., the focal adhesion complexes were concentrated in certain areas of the cell surface edges. Cells with non-polar focal adhesion complexes were defined as cells having more than 75% of their cell surface edges with focal adhesion complexes.

Actin Structure Assay

Cells were cultured and permeabilized as above, then stained with 0.25 μg/ml phalloidin-TRITC (Sigma) to detect actin. Photographs were taken under a fluorescence microscope at a magnification of 200x. Cells with polar actin filaments were defined as cells that had longitudinal actin filaments from one protrusion to another.

Results

In order to study MKP-1 function, human MKP-1 was overexpressed in rat pulmonary arterial smooth muscle cells (SMC). The expression of MKP-1 in transfected SMCs

was assessed by Northern blot hybridization analysis. Smooth muscle cells transfected with wild type MKP-1 or C258S had 4-fold (MKP-1) and 2.5-fold (C2583/MKP-1) higher expression than the non-transfected control cells.

SMC overexpressing MKP-1 (i.e., "MKP-1 cells") exhibited enhanced adhesion to type I collagen coated on tissue culture plates, compared to control SMC. As shown in Fig. 1, thirty minutes after plating, the number of MKP-1 cells adhering to type I collagen was more than twice the number of control SMC adhering to collagen.

Cell spreading in response to collagen was also increased in MKP-1 cells as compared to control SMC. Fig. 2 shows cells photographed under a phase-contrast microscope at 100x magnification 30, 60, 120 and 240 minutes after plating on collagen-coated tissue culture plates. Most of the MKP-1 cells were not light reflective 30 minutes after plating, indicating that most of the cells were spreading. In contrast, most of the control SMC were light reflective at this time, indicating that they were not spreading. As shown in Fig. 3, the percentage of cells which were spreading was approximately 6 fold higher in MKP-1 cells than in control SMC 30 minutes after plating. These results correlate with the enhanced adhesion to collagen observed in MKP-1 cells.

The MKP-1 cells reached a flat epithelial-like morphology by 120 minutes after plating. The control SMC took longer to reach this stage, with most of the cells exhibiting a flat morphology 240 min after plating. (Fig. 2). No significant difference in spreading was observed between MKP-1 cells and control SMC when the cells were plated on fibronectin coated dishes.

To further study the effects of MKP-1 overexpression, haptotaxis was measured in MKP-1 cells

and control SMC in a modified Boyden chamber assay. Bornfeldt et al., *J. Clin. Invest.* 93:1266-1274 (1994). In this assay, the top chamber contained the cells, and the bottom chamber contained DMEM with 0-10 ng/ml of the chemoattractant, Platelet Derived Growth Factor (PDGF). A filter separating the two chambers was coated on the bottom side with type I collagen. As shown in Fig. 4, MKP-1 cells showed a marked increase over control SMC in directional migration toward immobilized type I collagen in the lower Boyden chamber, in the presence or absence of PDGF.

In order to test whether MKP-1 plays a role in the migration of cell types other than SMC, NIH 3T3 fibroblasts were transfected with DNA encoding an intact MKP-1 gene. The resulting cell line, 3T3/MKP-1, overexpresses MKP-1, as demonstrated by Northern blotting and hybridization to a MKP-1 probe. The 3T3/MKP-1 cell line was tested in the Boyden chamber assay described above. As shown in Fig. 5, 3T3/MKP-1 cells, like MKP-1 cells, demonstrated increased migration toward collagen, compared to control cells.

The enhanced migration observed in MKP-1 cells may be due to the interaction of type I collagen with integrins, which are receptors for collagen, on the surface of the cells. The extracellular matrix (ECM) facilitates cellular adhesion and migration through interactions with cell surface receptors such as the integrins. The type I collagen signal can be mediated by the integrins $\alpha 2\beta 1$ (the collagen receptor) and $\alpha 3\beta 1$ (the ECM II receptor). Yoshinaga et al., *Melanoma Res.* 3:435-441 (1993); Keely et al., *J. Cell Sci.* (1995). Therefore, the role of these integrins in the migration of MKP-1 cells in response to collagen was analyzed. MKP-1 cells and control SMC were preincubated with antibodies specific for the $\alpha 2\beta 1$ or $\alpha 3\beta 1$ integrins as

described in Materials and Methods, and then tested in the Boyden chamber assay. The number of migrating cells was then calculated by counting cells in a 3x High Power Field (3x HPF). As shown in Fig. 6, antibodies specific for either $\alpha 2\beta 1$ or $\alpha 3\beta 1$ inhibited migration of MKP-1 cells by approximately 50% ($p<0.01$), while incubation with antibodies specific for the vitronectin receptor (VNR) had no inhibitory effect on migration. These results indicate that the $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins are involved in migration of MKP-1 cells in response to collagen.

Chemotactic factors stimulate cellular migration through pathways that are independent of the integrins. Rankin et al., *J. Biol. Chem.* 271:7829-7834 (1996). The effect of MKP-1 overexpression on chemoattractant-induced cellular migration was therefore studied using the Boyden chamber assay, and the results are shown in Fig. 7. In these experiments, PDGF was used as the chemoattractant, and no collagen was present. The number of MKP-1 cells migrating in response to PDGF was less than 20% of control SMC at all concentrations of PDGF tested (5, 10, and 20 ng/ml). The number of C258S/MKP-1 cells migrating was much greater, and increased with increasing concentration of PDGF. These results indicate that MKP-1 overexpression inhibits PDGF-induced migration. Therefore, MKP-1 is involved in both integrin-mediated and PDGF-mediated cellular migration.

Activation of integrins by the ECM leads to the recruitment and tyrosine phosphorylation of focal adhesion kinase (FAK), leading to the organization of FAK-associated cytoskeletal proteins and the formation of focal adhesion complexes. Parsons et al., *J. Cell. Sci. Suppl.* 18:109-113 (1994). To determine if MKP-1 is involved in this process, FAK and its associated cytoskeletal proteins were examined in MKP-1 cells.

Figs. 8A and 8B show the results of experiments in which cells were immunoprecipitated using either anti-phosphotyrosine (α-PY) or anti-FAK (α-FAK) antibodies, and Western blotted using anti-FAK antibodies. As shown in Fig. 8B, MKP-1 cells, C258S/MKP-1 cells and control SMC contain similar amounts of FAK. However, while control SMC and C258S/MKP-1 cells contained considerable amounts of tyrosine phosphorylated FAK, MKP-1 cells contained only about 20% of the amount found in control SMC. C258S/MKP-1 cells contained 140% of the amount of tyrosine phosphorylated FAK found in control SMC. Similar results were obtained when anti-FAK antibodies were used for immunoprecipitation and anti-phosphotyrosine antibodies were used on Western blots. In contrast to the difference between the amounts of tyrosine phosphorylated FAK in MKP-1 and control cells, the amounts of the cytoskeletal proteins paxillin, vinculin and α-actin were similar in all of the cell types tested (Figs. 8C-E).

A decrease in the amount of tyrosine phosphorylated FAK in MKP-1 cells can therefore be correlated with a decrease in migration of MKP-1 cells, and an increase in migration of C258S/MKP-1 cells, in response to PDGF. These results suggest that a reduction in FAK tyrosine phosphorylation contributes to the alteration of migration in MKP-1 cells. Cell migration has a biphasic relationship with adhesion force; adhesion force at a medial range is optimal for maximal migration speed. Lauffenburger et al., *Cell* 84:359-369 (1996); Mitchison et al., *Cell* 84:371-379 (1996); DiMilla et al., *J. Cell. Biol.* 122:729-737 (1993). Because FAK deficiency and high levels of FAK tyrosine phosphorylation have both been shown to inhibit cell motility, it is possible that the dramatic reduction in FAK tyrosine phosphorylation decreases MKP-1 cell

migration, while type I collagen mediated integrin activation may partially restore tyrosine phosphorylation of FAK, and promote cellular migration.

To test whether the cellular processes for migration are altered by MKP-1 overexpression, focal adhesion complexes were examined by immunostaining of the focal adhesion molecule vinculin. As shown in Fig. 9, the focal adhesion complexes in most of the control SMC had a polar distribution (94% of the cells examined), primarily localized at the edges of the cells. In contrast, the focal adhesion complexes in MKP-1 cells appeared to be stained less intensely and distributed evenly over the edges of the cells (90% of the cells examined). The focal adhesion complexes in C258S/MKP-1 cells were similar to those observed in control SMC. Similar results were obtained on immunostaining of paxillin, another focal adhesion component.

The effect of MKP-1 overexpression on actin structure was also examined. As shown in Fig. 10, control SMC exhibited a polar distribution of actin filaments. In contrast, in MKP-1 cells, the actin filaments were organized in a nonpolar, round to polygonal fashion (95% of the cells examined). The actin organization observed in C258S/MKP-1 cells was similar to that seen in the control SMC. These results indicate that the reduction in FAK tyrosine phosphorylation in MKP-1 cells may change the downstream cytoskeletal organization so that it is less polar. Such a reduced polarity may account for the observed alterations in cellular migration in MKP-1 overexpressing cells.

MKP-1 overexpression may exert its effects by inhibiting MAP kinase activity, thus altering the expression of a tyrosine kinase or phosphatase which regulates the tyrosine phosphorylation of FAK. The alteration of tyrosine phosphorylation of FAK and the

altered organization of focal adhesion complexes and actin filaments induced by MKP-1 overexpression may be responsible for changes in SMC adhesion and migration.

Example I: Screening Assays for Inhibitors of MKP-1 Activity

Screening assays can be used to identify agents which modulates, i.e., increase or decrease the expression or function of MKP-1. In one such assay, a cell-free preparation of MKP-1 is contacted with a test compound. MKP-1 activity is then detected in the preparation, for example, by using a substrate of MKP-1's phosphatase activity. An alteration in MKP-1 activity in the presence of the test compound indicates that the test compound is potentially useful for modulating migration of vascular smooth muscle cells.

Cell-based assays can also be used to screen for compounds which modulate migration of vascular smooth muscle cells. In one such method, a MKP-1-expressing cell is contacted with a test compound. MKP-1 expression is detected in the cell, using, for example, an antibody-based or nucleic acid hybridization assay. An alteration in MKP-1 activity in the presence of the test compound indicates that the test compound is potentially useful for modulating migration of vascular smooth muscle cells.

Example II: Therapeutic Administration of Inhibitors of MKP-1

Known inhibitors of MKP-1, such as sodium vanadate or antibodies specific for the $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins, or any agent identified by any of the above-mentioned screening assays, can be administered to treat conditions characterized by cellular migration. Other agents which can be used to inhibit MKP-1 function or expression

include antibodies specific for MKP-1, ribozymes specific for MKP-1 RNA, and antisense RNA molecules or oligonucleotides which hybridize specifically to the MKP-1 gene or mRNA.

The antibodies used can be polyclonal or monoclonal. Antibody fragments, such as Fab or $F(ab)_2$ fragments, can also be used. The antibody is preferably humanized.

The antisense molecules can be either DNA or RNA. These molecules can be directly introduced into the cells. Alternatively, they can be administered via an expression vector which enters the target smooth muscle cells and expresses the antisense molecules.

Ribozymes are RNA molecules that bind to RNA molecules and cleave them. Ribozymes can be used to inhibit MKP-1 expression in order to treat a condition characterized by cellular adhesion and migration.

In order to treat a condition associated with cellular migration, a patient with such a condition is first identified. Conditions characterized by cellular migration include post-angioplasty restenosis, transplant arteriopathy, graft stenosis and chronic atherosclerosis. The inhibitor can be administered as a therapeutic composition including one or more compounds and a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are biologically compatible vehicles which are suitable for administration to an animal: e.g., physiological saline. A therapeutically effective amount of the composition is administered to the patient. A therapeutically effective amount is an amount which is capable of producing a medically desirable result in a treated animal, e.g., inhibition of expression of the target gene, or inhibition of MKP-1 enzymatic activity to the extent necessary to inhibit the target cellular activity, such as adhesion or migration.

Parenteral administration, such as intravenous, intrapulmonary, intranasal, subcutaneous, intramuscular, and intraperitoneal delivery routes may be used to deliver the compound, with intravenous administration being the preferred route. Dosages for any one patient depend upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently.

Example III: Screening Assays for Agents that Increase MKP-1 Activity

SMC such as vascular SMC or other cells in which the endogenous MKP-1 gene is present (but the expression of which is down-regulated) can be used as a screening tool to identify compounds or treatment strategies which induce an increase in expression of the MKP-1 gene. Other cell types with intact but unexpressed MKP-1 genes would also be potentially useful in this screening assay. The cells are contacted *in vitro* with the candidate compounds, and the amount of MKP-1 expression determined using known methods, e.g., a hybridization assay (e.g., Northern analysis) or an immunoassay to detect MKP-1 polypeptides. If a given compound is found to stimulate MKP-1 expression, it is then further tested to see whether treatment with the compound inhibits SMC chemotaxis and in the Boyden chamber assay described above. A compound effective both in stimulating MKP-1 expression and in preventing SMC migration and adhesion is useful for the treatment of conditions characterized by unwanted SMC migration and adhesion.

Example 4: Treatment with Agents that Increase MKP-1 Expression

As discussed above, increasing the level of MKP-1 expression in a SMC cell correlates with a decrease in its ability to migrate toward PDGF, a chemoattractant. Thus, it is expected that treating a patient with MKP-1, or a biologically active fragment of MKP-1 (i.e., a fragment able to dephosphorylate MAP kinase) of MKP-1, will inhibit unwanted migration of SMC. A useful treatment protocol will therefore be a method such as intravenous injection of the protein in a pharmaceutically acceptable solution in a dosage of 0.001 to 100 mg/kg/day, with the most beneficial range to be determined using routine pharmacological methods. Alternative routes of delivery would also be acceptable, such as intramuscular or subcutaneous injection, injection directly into the tumor site, or implantation of a device containing a slow-release formulation. The MKP-1 protein or nucleic acid encoding MKP-1 could be incorporated into liposomes or another form of carrier which permits substantial amounts of the protein to pass through the cell membrane. Liposomes would also help protect the protein from proteolytic degradation while in the bloodstream.

Example 5: Genetic therapy

An expression vector encoding MKP-1 can be introduced into SMC, thereby increasing the production of MKP-1 in the transfected cells, and decreasing the abilities of these cells to migrate. The transfected cells are also shown above to have decreased migrating ability compared to control cells. This evidence indicates that the MKP-1 DNA will be useful for genetic therapy to help control conditions characterized by unwanted migration of SMC. Standard methods of gene therapy may be employed: e.g., as described in Friedmann, *Therapy for Genetic Disease*, T. Friedman

(ed.), Oxford Univ. Press, 1991, pp.105-121. Virus or plasmids containing a copy of the MKD-1 cDNA linked to expression control sequences which permit expression in the target cell would be introduced into the patient, either locally at the site of unwanted SMC migration or systemically. If the transfected DNA encoding MKP-1 is not stably incorporated into the genome of each of the targeted cells, the treatment may have to be repeated periodically.

Other Embodiments

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

What is claimed is:

1. A method of inhibiting the migration of vascular smooth muscle cells in a vertebrate, comprising:

identifying a vertebrate suspected of having a condition characterized by unwanted migration of vascular smooth muscle cells;

providing an agent that increases expression or activity of MKP-1 in vascular smooth muscle cells; and

administering to the vertebrate an amount of the agent sufficient to inhibit said migration.

2. The method of claim 1, wherein said agent comprises a naturally occurring nucleic acid sequence encoding MKP-1.

3. The method of claim 1, wherein said vertebrate is a mammal.

4. The method of claim 1, wherein said vertebrate is a human.

5. The method of claim 1, wherein said condition is post-angioplasty restenosis.

6. The method of claim 1, wherein said condition is transplant arteriopathy.

7. The method of claim 1, wherein said condition is graft stenosis.

8. The method of claim 1, wherein said condition is chronic atherosclerosis.

9. A method of inhibiting the migration of cells in a vertebrate by administering an agent selected from

the group consisting of antibodies to $\alpha 2\beta 1$ integrin and antibodies to $\alpha 3\beta 1$ integrin.

10. A method for identifying a compound which inhibits migration of vascular smooth muscle cells, comprising:

providing a cell-free preparation of MKP-1,

contacting the MKP-1 with a test compound;

detecting MKP-1 activity;

determining whether the test compound increases MKP-1 activity, wherein an increase in MKP-1 activity in the presence of the test compound indicates that the test compound inhibits migration of vascular smooth muscle cells.

11. The method of claim 10, wherein the MKP-1 activity is detected using a substrate of MKP-1's comprising a phosphorylated tyrosine or threonine residue.

12. A method for identifying a compound which inhibits migration of vascular smooth muscle cells, comprising:

providing a cell which expresses MKP-1;

contacting the cell with a test compound;

detecting MKP-1 expression in the cell; and

determining whether the level of MKP-1 expression is increased in the cell in the presence of the test compound, wherein an increase in MKP-1 activity in the presence of the test compound indicates that the test compound inhibits migration of vascular smooth muscle cells.

13. The method of claim 16, wherein MKP-1 expression is detected using an antibody-based assay.

14. The method of claim 16, wherein MKP-1 expression is detected using a nucleic acid hybridization assay.

15. A method for identifying compounds which inhibit migragtion of vascular smooth muscle cells, comprising:

providing a first and a second sample of vascular smooth muscle cells;

treating said first sample with a test compound; and

comparing the level of expression of MKP-1 in said first sample with the level in said second sample, a higher level of expression in said first sample being an indication that said test compound inhibits migration of smooth muscle cell.

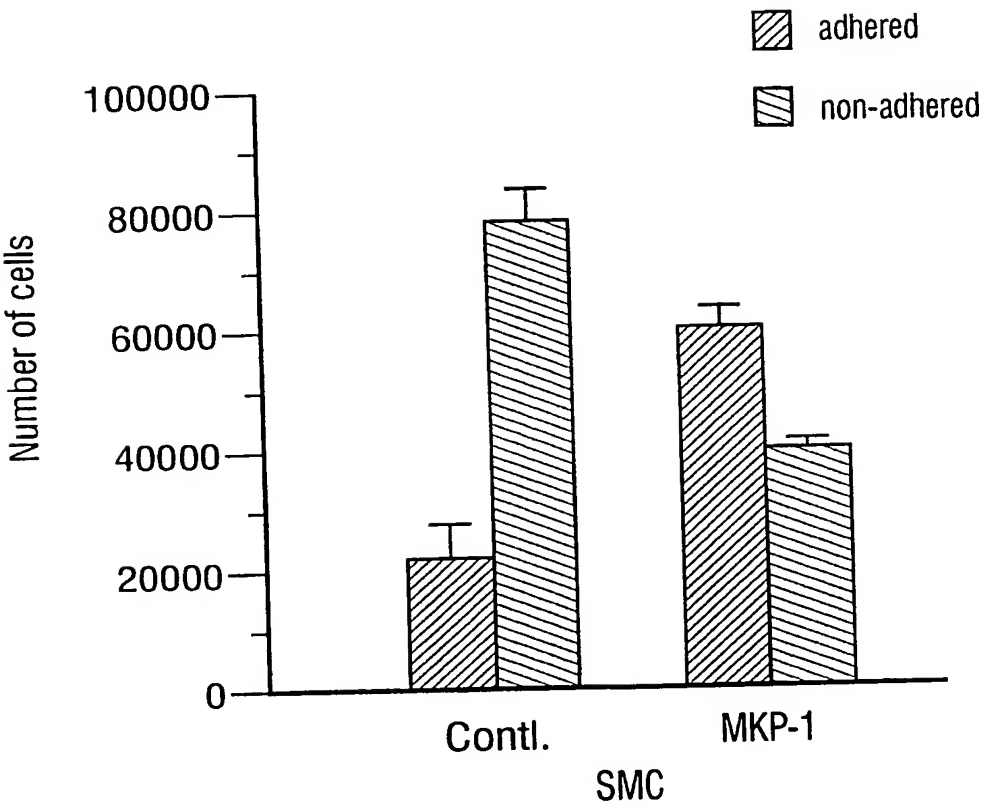
adhered
non-adhered
100000
80000
60000
40000
20000
0
Number of cells
Contl.
MKP-1
SMC

Figure 1

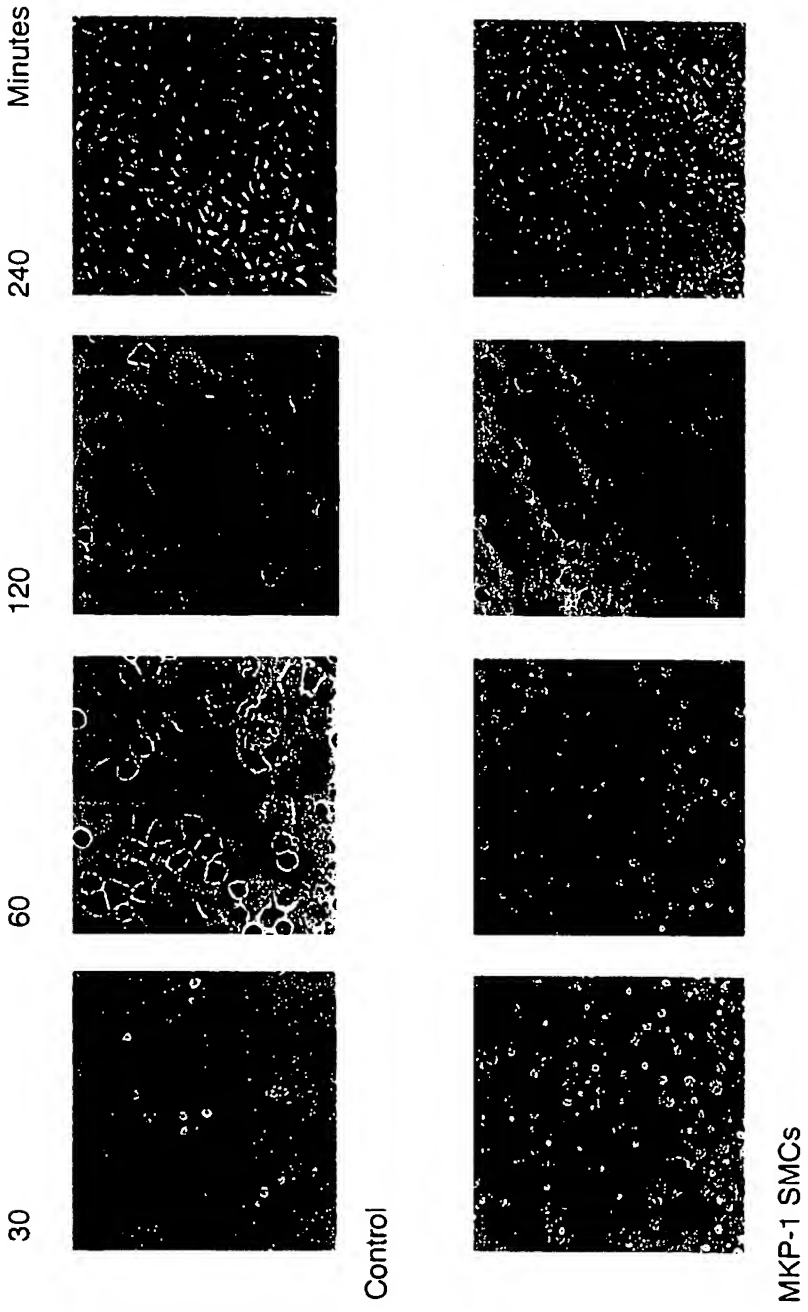

Fig. 2

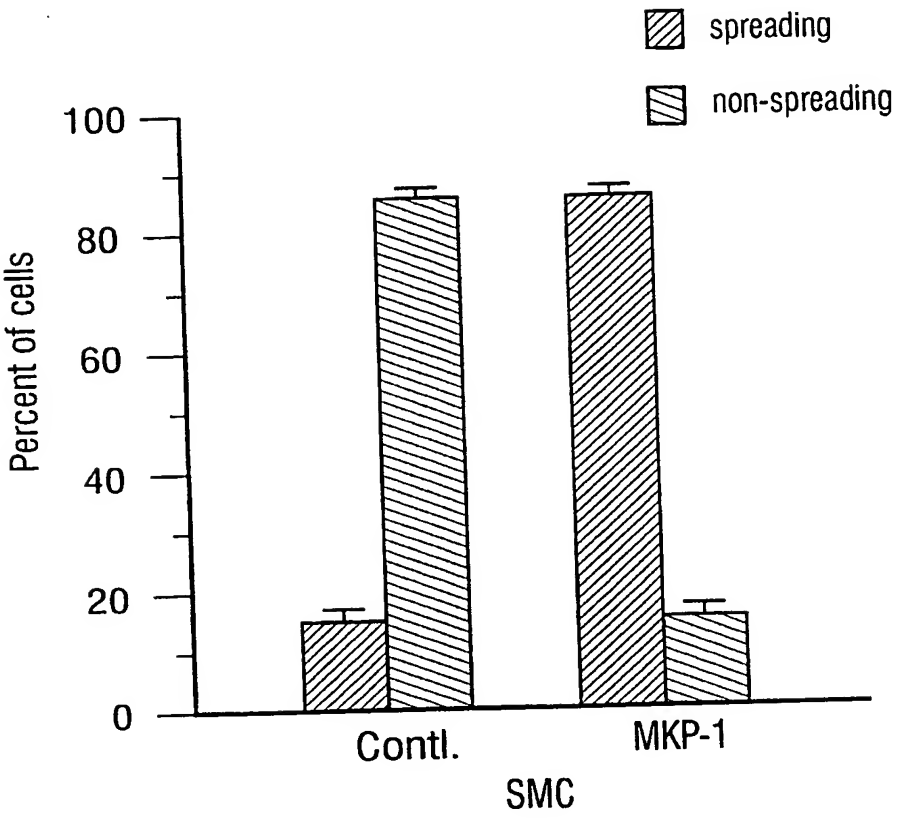
spreading
non-spreading
100
80
60
40
20
0
Percent of cells
Contl.
MKP-1
SMC

Figure 3

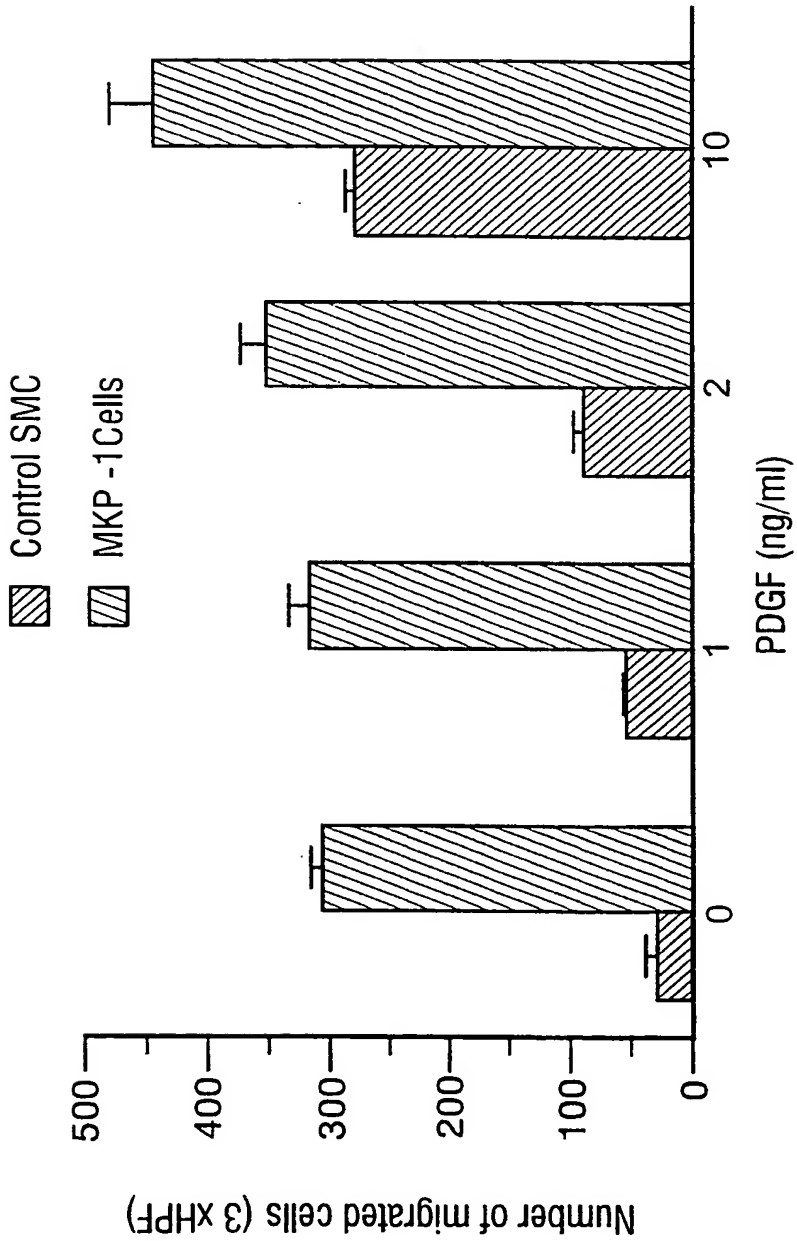
Control SMC
MKP -1Cells
Number of migrated cells (3 xHPF)
500
400
300
200
100
0
0
1
2
10
PDGF (ng/ml)

Figure 4

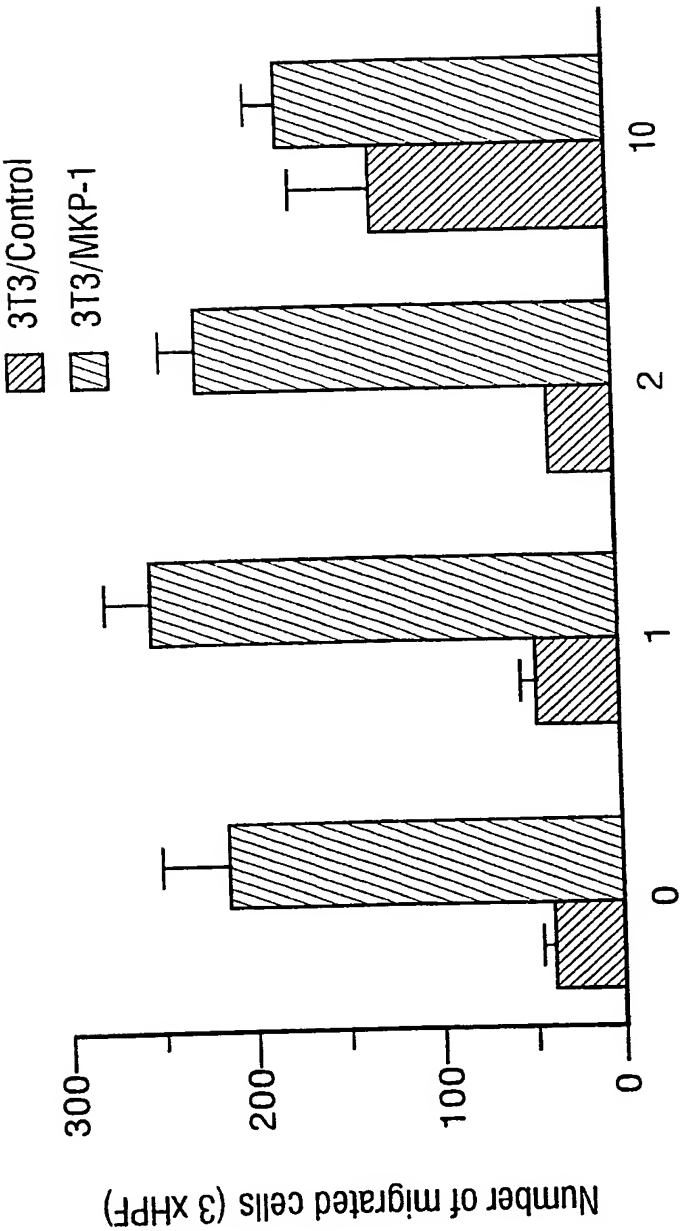
3T3/Control
3T3/MKP-1
Number of migrated cells (3 xHPF)
300
200
100
0
0
1
2
10
PDGF (ng/ml)

Figure 5

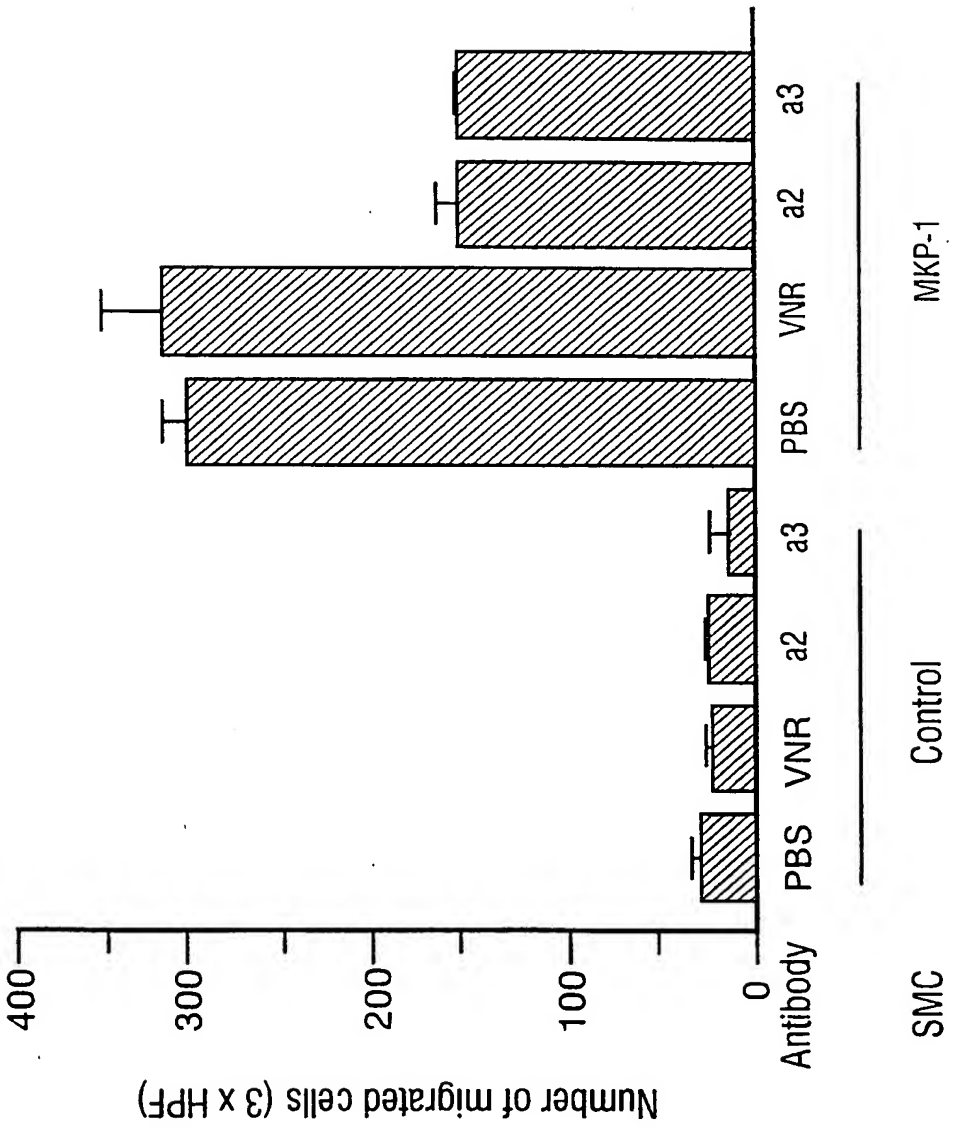
Number of migrated cells (3 x HPF)
400
300
200
100
0
Antibody
PBS
VNR
a2
a3
PBS
VNR
a2
a3
SMC
Control
MKP-1

Figure 6

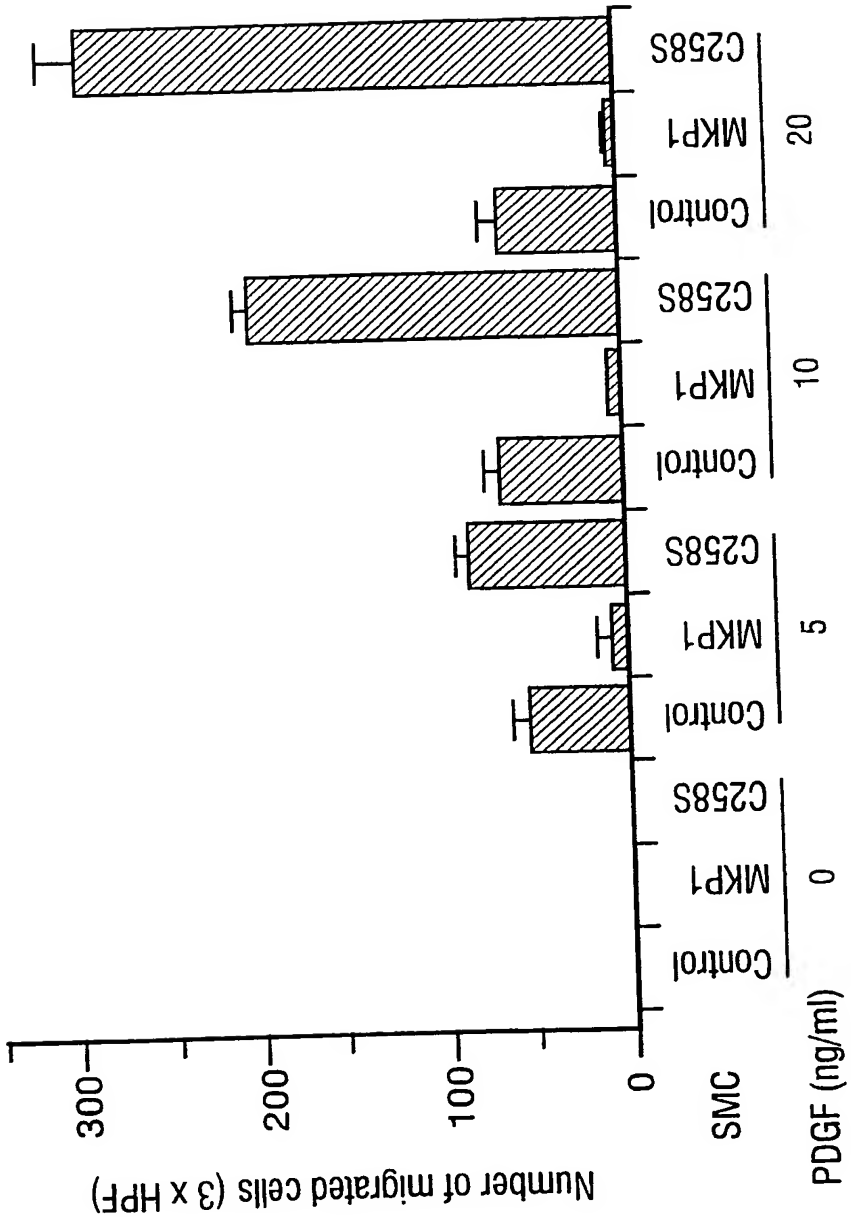
Number of migrated cells (3 x HPF)
300
200
100
0
SMC
PDGF (ng/ml)
0
5
10
20
Control
MKP1
C258S

Figure 7

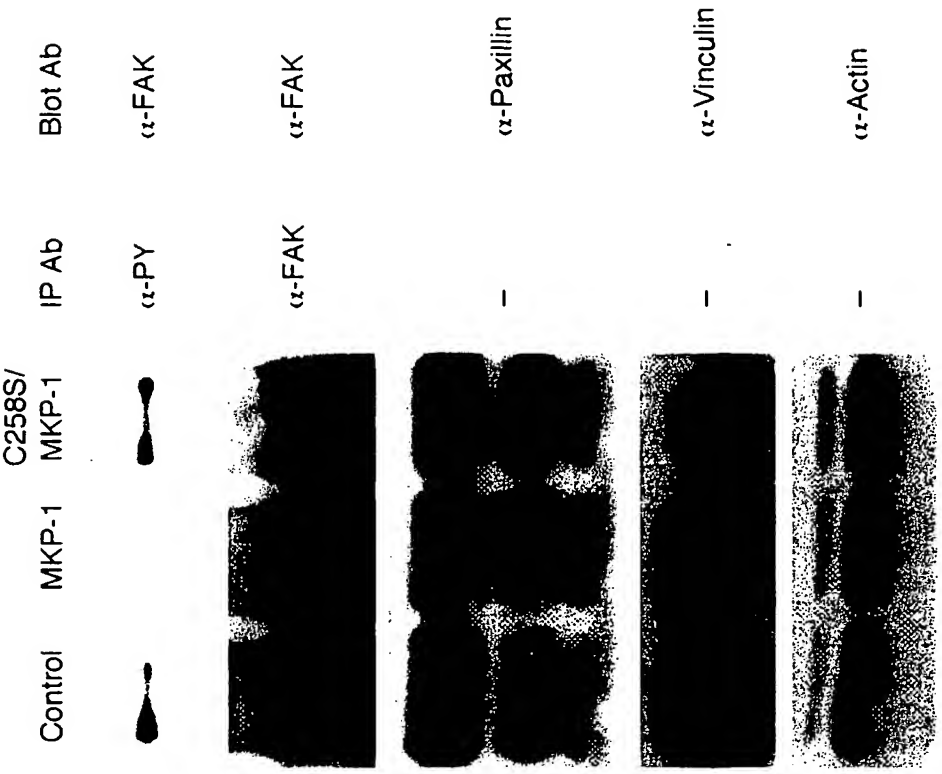

Fig. 8A

Fig. 8B

Fig. 8C

Fig. 8D

Fig. 8E

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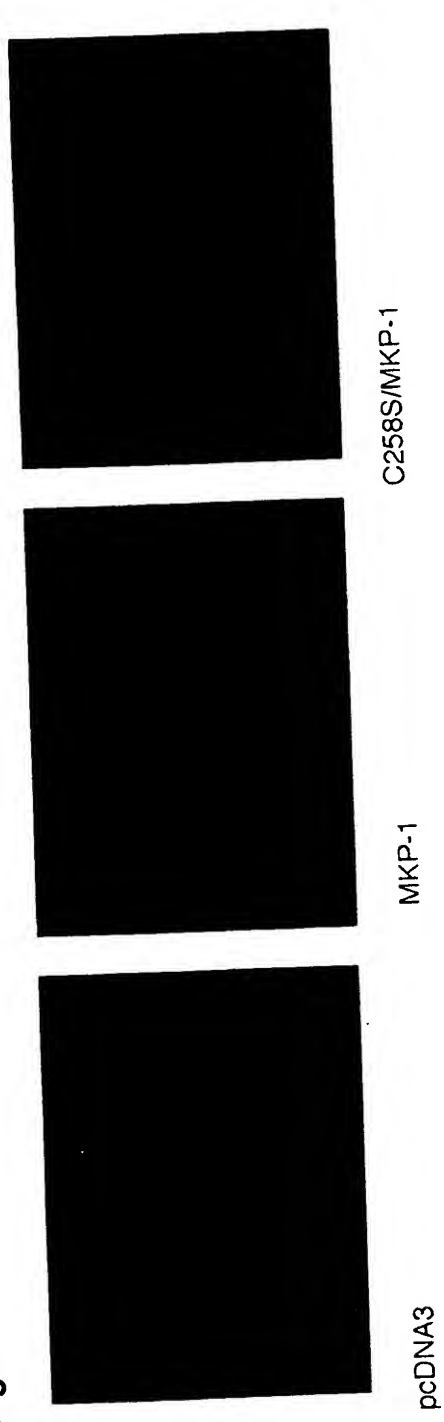

Fig. 9

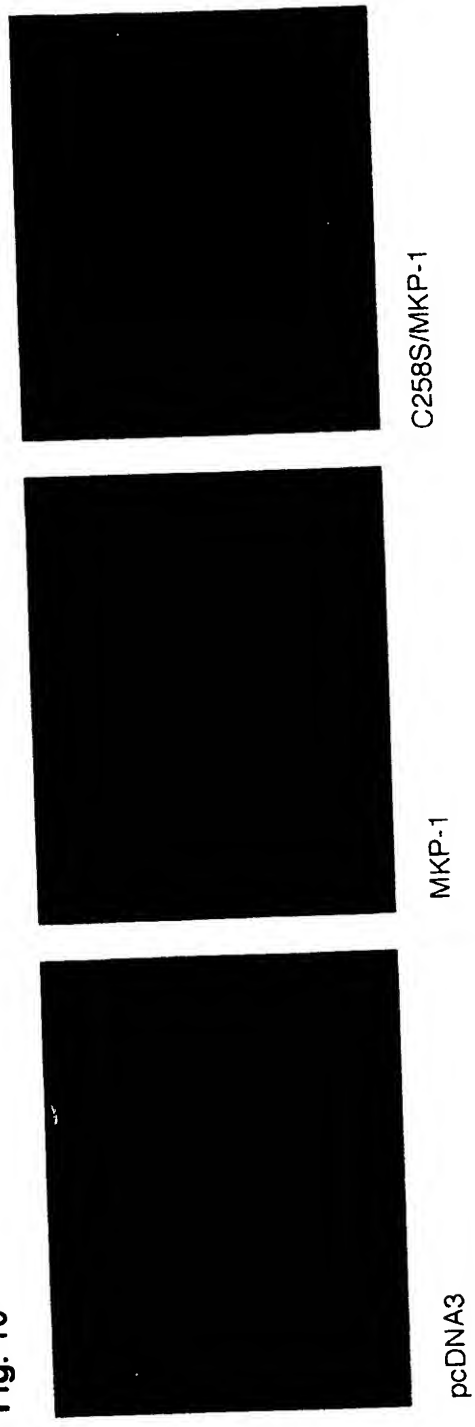

Fig. 10

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INTERNATIONAL SEARCH REPORT

Internati Application No
PCT/US 97/20744

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/46 A61K39/395 G01N33/573

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LAI ET AL: "POTENTIAL ROLES OF TYROSINE PHOSPHATASE MKP-1 IN THE PROLIFERATION OF RAT VASCULAR SMOOTH MUSCLE CELLS" FASEB JOURNAL, vol. 10, no. 6, 30 April 1996, page A1139 XP002058892 see abstract 810 ---	1-8, 10-15
X	LAI ET AL: "MITOGEN-ACTIVATED PROTEIN KINASE PHOSPHATASE-1 IN RAT ARTERIAL SMOOTH MUSCLE CELL PROLIFERATION" JOURNAL OF CLINICAL INVESTIGATION, vol. 98, no. 7, October 1996, pages 1560-1567, XP002058893 cited in the application see the whole document --- -/--	1-8, 10-15

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
13 March 1998	08.04.98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Sitch, W

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Internati Application No
PCT/US 97/20744

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SKINNER ET AL: "DYNAMIC EXPRESSION OF ALPHA1BETA1 AND ALPHA2BETA1 INTEGRIN RECEPTORS BY HUMAN VASCULAR SMOOTH MUSCLE CELLS" AMERICAN JOURNAL OF PATHOLOGY, vol. 145, 1994, pages 1070-1081, XP002058894 see page 1070 see abstract ---	9
X	JONES ET AL: "LIGAND OCCUPANCY OF THE ALPHAVBETA3 INTEGRIN IS NECESSARY FOR SMOOTH MUSCLE CELLS TO MIGRATE IN RESPONSE TO INSULIN-LIKE GROWTH FACTOR I" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES,USA, vol. 93, March 1996, pages 2482-2487, XP002058895 see abstract see page 2482 ---	9
P,X	LAI ET AL: "MKP-1 REGULATES VASCULAR SMOOTH MUSCLE CELL MIGRATION" MOLECULAR BIOLOGY OF THE CELL, vol. 7, December 1996, page 234A XP002058896 see abstract 1361 -----	1-15

1

INTERNATIONAL SEARCH REPORT

Int tional application No.
PCT/US 97/ 20744

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 1-9
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 1-9 is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

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